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ANTITUMOR EFFECT OF *BIFIDOBACTERIUM INFANTIS* IN MICE*1

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Bifidobacterium infantis is a gram-positive anaerobic bacterium isolated from the feces of human infants. Studies were made on its antitumor effect on cells of an ascites Meth-A tumor induced in a BALB/c mouse with 3-methylcholanthrene. The effect of intraregional injection of this bacteria on subcutaneously transplanted tumor (25×10^3) produced a complete regression in all the mice, when treatment with *B. infantis* was started from one day after tumor inoculation for 6 times, where control mice were all dead. The majority of surviving mice rejected the rechallenge of the tumor by this treatment. When tumor cells mixed with *B. infantis* were inoculated subcutaneously into mice, tumor did not grow in a majority of the recipient mice, but these surviving mice accepted rechallenge of the tumor. Intraperitoneal injection of *B. infantis* against intraperitoneally transplanted tumor also exhibited a remarkable antitumor effect. The effect decreased as the dosage of *B. infantis* decreased.

Certain microörganisms, represented by BCG,[18,19] *Corynebacterium parvum*,[2,5] and *Streptococcus haemolyticus*,[9,15] or cellular components[1,17] of some of these and of other bacteria inhibit tumor growth when injected into tumor or at the site remote from the tumor, inducing a local inflammatory reaction or increasing the host's immunological resistance against cancer. Application of these microörganisms to cancer therapy had often been based on clinical implications[3,6] such as spontaneous regression of cancer in a patient infected with the bacteria and resistance to carcinogenesis in a patient who had suffered from infectious diseases. The less pathogenic bacteria predominantly present in animals and in humans seem to play a role in the host-defense mechanisms, while antitumor effect of such bacteria has been little examined. One of such bacteria, *Propionibacterium acnes*, which was isolated from bone marrow of a healthy man,[7] has been tested for its antitumor effect in experimental animals.[8]

Bifidobacterium is a gram-positive anaerobic organism that is predominant in human intestine.[4,10] It has been shown that this organism is non-pathogenic and caused few side effects when injected into animals irrespective of its administration route. Furthermore its presence in human infants is thought to be an indication of health.[12] Therefore it is of interest to see whether this bacterium shows antitumor effect like the already known antitumor microörganisms. From this view, we examined the antitumor effect of *Bifidobacterium* against a mouse tumor transplanted in syngeneic animals.

The present report shows that two strains of *Bifidobacterium* of human origin exhibited a marked antitumor effect, especially when the bacteria were injected into the tumor

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region, and that the non-living preparation of the bacteria is as effective as the living bacteria.

Materials and Methods

Animals Male and female BALB/c mice of 8 to 10 weeks old were used throughout. They were obtained from the Institute of Medical Science, University of Tokyo, and maintained on a pellet diet (CE-2, CLEA Japan Inc., Tokyo).

Tumor Cells Meth-A ascites tumor cells, originating from a sarcoma induced with 3-methylcholanthrene in a BALB/c mouse,[14] were donated by the Sloan-Kettering Institute for Cancer Research (New York, U.S.A.). They were maintained in our laboratory by weekly intraperitoneal passage into BALB/c mice. Ascites tumor cells were washed 3 times with isotonic phosphate-buffered saline (PBS), pH 7.2, and suspended in PBS.

Microörganisms *Bifidobacterium infantis* and *Bifidobacterium adolescentis a* were donated by Dr. T. Mitsuoka, the Institute of Physical and Chemical Research, Wako, and maintained in our laboratory. For animal experiments, bacteria were cultured in Briggs liver broth for 24 hr under anaerobic conditions,[11] washed with PBS, and suspended in PBS. Non-living bacterial preparations were obtained by keeping the living bacteria under aerobic conditions for 3 to 7 days, and were certified for loss of growth ability.

A lyophilized preparation of penicillin-treated *Streptococcus haemolyticus*[13,16] was donated by Chugai Pharmaceutical Co., Tokyo. One KE (clinical unit) of this preparation corresponds to 0.1 mg (10^8 cells) of dry bacteria.

Test of Antitumor Effect of Microörganisms A volume of 0.1 ml of a suspension containing a known number of Meth-A cells was inoculated intraperitoneally or subcutaneously into BALB/c mice, with or without bacteria. In the latter case, the mice were repeatedly injected with bacteria at the tumor inoculated site. Two KE of OK-432 or 10^9 *Bifidobacterium* cells were used for a single dose into the tumor. The size of the tumor with overlying skin was periodically measured with a caliper and expressed as the mean of two diameters. The significance of the difference in period of survival of experimental and control animals was estimated by Student's *t*-test.

Results

Throughout the experiments, the antitumor effect of *Bifidobacterium* to BALB/c Meth-A sarcoma cells was comparatively studied with that of OK-432, whose antitumor effect has been established for animal tumors.[16] Since it has been known that OK-432 was effective to the tumors at the doses over 2 KE/mouse as a single dose, 2 KE (0.2 mg) of OK-432 and the equivalent weight (0.25 mg, 10^9 cells) of *Bifidobacterium* were used for a single injection dose in the majority of the experiments.

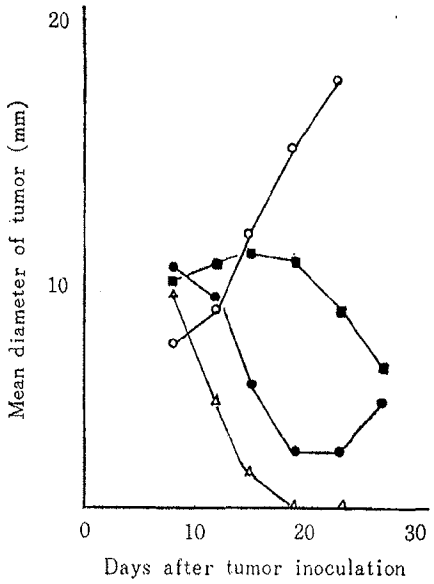

Fig. 1. Effect of intraregional injection of *B. infantis* and OK-432 on the growth of Meth-A cells inoculated subcutaneously

Meth-A cells (25×10^3) were inoculated into male BALB/c mice and bacterial preparations were injected intraregionally on days 1, 2, 3, 5, 6, and 7. All survivors at day 50 were free of tumor.
○——○ PBS, ●——● *B. infantis*, △——△ killed *B. infantis*, ■——■ OK-432

Effect of Intraregional Injection of Bacteria on Subcutaneously Transplanted Meth-A Tumor Effect of *B. infantis* and *B. adolescentis* was tested on mice with a small (25×10^3 cells) and large (500×10^3 cells) inoculum sizes of Meth-A cells with different time schedules of the treatment, and the results are shown in Fig. 1 and Table I. As illustrated in Fig. 1, no significant difference in tumor sizes in mice inoculated with 25×10^3 tumor cells, measured on the 8th day, was observed between control given PBS and the

Table I. Effect of Intraregional Injection of Bacteria on Subcutaneously Inoculated Meth-A Tumor Cells

No. of inoculated tumor cells ($\times 10^3$)	Material for treatment[a]	Day of treatment	No. of mice dying with tumor/No. tested (% survivors)[b]		Survival days of dead mice (mean±SE)
25	PBS	2, 4, 6, 8,	12/12	(0)	30.5±1.8
	B. infantis		4/16	(75)	35.3±2.8
	Killed *B. infantis*		5/15	(67)	28.6±1.2
	B. adolescentis		5/15	(67)	33.4±1.2
	Killed *B. adolescentis*		3/7	(43)	34.0±3.0
	OK-432		4/15	(73)	30.5±1.5
25	PBS	3, 5, 7, 9	7/7	(0)	28.1±1.1
	B. infantis		4/7	(43)	31.8±3.4
	OK-432		6/7	(14)	30.6±2.5
	B. infantis	7, 9, 11, 13	5/7	(29)	29.4±1.6
	OK-432		6/6	(0)	31.3±2.0
500	PBS	2, 4, 6, 8	7/7	(0)	23.6±1.0
	B. infantis		6/8	(25)	39.2±6.5
	OK-432		7/7	(0)	29.6±4.2

a) A known number of Meth-A cells were inoculated subcutaneously into male BALB/c mice, and the mice were repeatedly injected with bacteria (10^9 cells/injection) or OK-432 (2 KE/injection) intraregionally on the days shown in the table.

b) All of the surviving mice were free of tumor on observation at day 50. Standard error (SE) was calculated when more than 3 mice died with tumor.

groups treated with bacteria. In later days, however, many of the established tumors underwent complete or partial regression in mice treated with *B. infantis*, killed *B. infantis*, *B. adolescentis*, or with OK-432, although in the control mice, tumors grew to kill the hosts. To a small inoculum size of Meth-A cells, the intraregional injections of *B. infantis* and *B. adolescentis*, and their killed preparations, were as effective as OK-432, as revealed from the result in Fig. 1 and from the number of survivors shown in Table I, when the treatment was started 1 or 2 days after tumor inoculation and repeated 4 or 6 times. Efficacy of the antitumor effect of the bacteria decreased, however, when the first injection of the bacteria was performed 3 or 7 days after tumor inoculation, or when mice had been inoculated with a large number of Meth-A cells.

Effect of Bacteria on Mixed Implantation of Meth-A Tumor A mixture of bacteria and Meth-A cells was inoculated subcutaneously into mice. As shown in Table II, tumor did not grow in a majority of the recipient

Table II. Suppression of Growth of Meth-A Tumor Injected with Bacteria

Material injected with tumor cells[a]	No. of mice dying with tumor/No. tested (% survivors)[b]	Survival days of dead mice (mean±SE)
PBS	8/8 (0)	43.9±2.9
B. infantis	3/9 (67)	53.7±4.3
Killed *B. infantis*	2/9 (78)	34, 34
OK-432	2/9 (78)	50, 53

a) PBS (phosphate-buffered saline) or cell suspension containing 25×10^3 Meth-A cells and 10^9 bacteria or 2 KE OK-432 was injected subcutaneously into male BALB/c mice.

b) All of the surviving mice were free of tumor on the observation at day 60. See the footnote to Table I.

mice inoculated with 25×10^3 tumor cells mixed with 10^9 of *B. infantis*. Killed *B. infantis* and OK-432 were also effective to Meth-A tumor cells.

Effect of Intraperitoneal Injection of Bacteria on Intraperitoneally Transplanted Meth-A Tumor Mice inoculated with Meth-A cells were treated with intraperi-

Table III. Effect of Intraperitoneal Injection of Bacteria on Intraperitoneally Inoculated Meth-A Tumor Cells

No. of inoculated tumor cells ($\times 10^3$)	Material for treatment[a]	No. of mice dying with tumor/No. tested (% survivors)[b]		Survival days of dead mice (mean±SE)
50	PBS	6/7	(14)	28.0±1.9
	B. infantis	0/7	(100)	
	Killed *B. infantis*	3/7	(57)	24.7±3.0
	OK-432	2/7	(71)	36, 39
100	PBS	6/6	(0)	31.7±4.9
	B. infantis	3/6	(50)	32.0±8.7
	OK-432	5/6	(17)	30.0±1.9
500	PBS	15/15	(0)	14.3±0.7
	B. infantis	16/17	(6)	18.6±0.8[c]
	OK-432	13/15	(13)	18.4±0.9[c]

a) The treatment was started one day after tumor inoculation and repeated 6 times during 7 days with 10^9 cells of *Bifidobacterium* or 2 KE of OK-432.
b) See footnote to Table I.
c) Statistical significance to the control by Student *t*-test: $P<0.01$

Table IV. Dose-response of *B. infantis* against Intraperitoneally Inoculated Meth-A Tumor Cells

Mateiral for treatment[a]	No. of bacteria injected	No. of mice dying with tumor/No. tested (% survivors)[b]		Survival days of dead mice (mean±SE)
PBS		14/14	(0)	25.8±1.6
B. infantis	4×10^7	12/15	(25)	33.9±1.5[c]
	2×10^8	10/14	(29)	30.5±2.5
	10^9	8/15	(47)	38.6±2.3[c]

a) The treatment was started 1 day after inoculation of 100×10^3 Meth-A cells and repeated 6 times during 7 days.
b) See footnote to Table I.
c) See footnote to Table III.

toneal injections of 10^9 cells of *Bifidobacterium*, 6 times daily for 1 week from the day after tumor inoculation. As shown in Table III, the antitumor effect of bacteria depended on the number of inoculated tumor cells. Injection of *B. infantis*, killed *B. infantis*, or OK-432 into mice inoculated with 50×10^3 Meth-A cells resulted in a marked suppression on tumor graft and many of the mice survived. By contrast, to mice which were inoculated with 500×10^3 tumor cells, injections of either bacterium were effective only to a small extent.

Table IV summarizes the dose-response in *B. infantis* treatment. With 6 injections of 10^9 cells of the bacteria into mice, which had been inoculated with 100×10^3 tumor cells, 47% of the mice were cured of tumor. However, with 1/5 and 1/25 dose of the above, the percentage of cured mice decreased to 29 and 20% of the tested mice, respectively.

Retransplantation of Meth-A Cells into Mice Cured from the Tumor In the preceding experiments, 2 groups of mice cured from tumor inoculation were obtained; group 1 of mice cured from tumor transplanted mixed with bacteria and group 2 of mice cured by therapeutic treatment of subcutaneous tumor. All these mice, as well as the non-treated control mice, accepted

subcutaneous rechallenge of 25×10^3 Meth-A cells. All 10 untreated control and 20 of group 1 mice died due to tumor growth, whereas 63 out of 67 of group 2 mice rejected the rechallenged Meth-A cells and survived.

Discussion

Two strains of *Bifidobacterium* isolated from a human exhibited a remarkable antitumor effect to Meth-A sarcoma cells transplanted into syngeneic BALB/c mice. Referring to the antitumor mechanisms presented for other bacteria, several possibilities can be considered: (1) Bacteria are cytotoxic to tumor cells, (2) inflammatory response may develop against the bacteria and tumor cells present in such a locus could be damaged nonspecifically as an innocent bystander, (3) injection of bacteria may produce an augmentation of host immune response specific to the tumor, or (4) the combination of the above mechanisms. The first possibility can be ruled out from the fact that bifidobacteria are not cytotoxic to tumor cells *in vitro* (unpublished data). The second one may be probable since tumor cells inoculated with the bacteria were suppressed in their growth, suggesting the role of an acute and local host-mediated reaction. However, in the therapeutic model, where the treatment with bacteria was undertaken the day after tumor inoculation, tumor grew for 1 week and then regressed. This finding may suggest the involvment of a host-mediated immunological response to tumor, at least to some extent, together with the nonspecific local reaction. This assumption could be substantiated from the finding that the mice that survived this experiment rejected the rechallenged tumor cells, indicating the acquirement of transplantation immunity to Meth-A cells. Furthermore, surviving mice which had been inoculated with tumor cells mixed with bacteria accepted rechallenge of the tumor cells and died, probably due to the insufficient antigenic stimulation with the first reaction. Taken together, destruction of a part of tumor cells by the local reaction induced by the bacteria and the following immunological stimulation with the tumor may account for the antitumor mechanism of the bacteria. Like *C. parvum* and *P. acnes*, killed *Bifidobacterium* was as effective as the living one, indicating the viability or growth of bacteria in the recipient animals could not be a predominant factor for its antitumor effect.

Further studies on the augmentation of immunological function in the host stimulated with *Bifidobacterium* will be reported elsewhere. The isolation and analysis of the effective component of *B. infantis* would be considered important as the next step, since BCG cell-wall skeleton[1] and *Nocardia rubra* cell-wall skeleton[17] have been shown to be as effective as and less toxic than the original bacteria.

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References

1) Azuma, I., Ribi, E., Meyer, B., Zbar, B., *J. Natl. Cancer Inst.*, **52**, 95～101 (1974).
2) Bomford, R., *Br. J. Cancer*, **32**, 551～557 (1975).
3) Busch, W., *Berlin Klin. Wochenschr.*, **5**, 137～139 (1868).
4) Cowan, S. T., Holt, J. G., Liston, J., Murray, R. G. E., Niver, C. F., Ravin, A. W., Stainier, R. Y., "Bergey's Mannual of Determinative Bacteriology," 8th Ed. (1974). Williams & Wilkins, Baltimore.
5) Halpern, B., Biozzi, G., Stiffel, C., Muton, D., *Nature* (*London*), **212**, 853～854 (1966).
6) Hattori, T., Ito, I., Hirata, K., *Gann*, **55**, 211～244 (1964).
7) Hattori, T., Mori, A., *Gann*, **64**, 7～14 (1973).
8) Ikeda, H., Yamagata, S., Toge, T., Hattori, T., *Gann*, **68**, 233～235 (1977).
9) Koshimura, S., Shimizu, R., Fujimura, A., Okamoto, H., *Gann*, **55**, 233～236 (1964).
10) Mitsuoka, T., *Jpn. J. Bacteriol.*, **24**, 261～280 (1969).
11) Mitsuoka, T., *Rinsho Kensa*, **18**, 1163～1172 (1974).
12) Negishi, A., *Jpn. J. Pedatr.*, **16**, 132～142 (1964).

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